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Monitoring Amphibian and Reptile Populations Using Environmental DNA Year 1 Report

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Monitoring Amphibian and Reptile Populations Using Environmental DNA

Technical report for Legacy Project 12-616 Year 1

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Abstract

Accurate information on the distribution of at-risk species is required for informed and cost-effective management actions and to prevent restrictions on military access and training. We developed a novel and efficient method for detecting two native at-risk aquatic species (Arizona treefrog, *Hyla wrightorum*; northern Mexican gartersnake, *Thamnophis eques megalops*) in and around Fort Huachuca, Arizona, using environmental DNA collected from water samples. Additionally, we applied this method to detect threats to these target species: an invasive amphibian (American bullfrog, *Lithobates catesbeianus*) and the amphibian pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). In this first year, we found that the eDNA technique worked excellently for the amphibians and the pathogenic fungus. The gartersnake was more difficult to detect and poses additional challenges for application of this technology.

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1. INTRODUCTION

Amphibians are among the most imperiled vertebrate taxa, declining more rapidly than birds and mammals, due in part to the dependence of many species on freshwater systems that are often highly modified (Ricciardi and Rasmussen 1999, Stuart et al. 2004). Recently, scientists have also come to recognize widespread declines in reptile populations (Gibbons et al. 2000). The presence of these threatened and endangered species on military lands can place restrictions on use of lands for readiness training. To avoid conflicts, the Department of Defense (DoD) actively monitors populations of listed and at-risk species; however, many aquatic species pose unique challenges for monitoring due to the difficulty of thoroughly surveying aquatic environments. Current technologies for detection of aquatic amphibians and reptiles include dip nets, seining, traps, and audio and visual surveys, all of which can have low detection probabilities and/or require high inputs of field time of trained technicians and disturbance to the aquatic environment.

Recent technological advances in the use of environmental DNA (eDNA) to detect species in water bodies indicate that eDNA can be used as an alternative or complementary method for inventory and monitoring of aquatic species (Ficetola et al. 2008, Goldberg et al. 2011, Jerde et al. 2011,Thomsen et al. 2012). Potential benefits of eDNA include high detection rates, lack of disturbance of sensitive species, reduced field efforts, and reduced species-specific training for field technicians. This technique has the potential to be highly efficient in that many species can be tested for in a single reaction once the protocol is optimized, reducing field time and increasing accuracy without any impact on sensitive species. The eDNA method has been demonstrated in multiple systems, but has not yet been applied systematically to monitor at-risk species.

Fort Huachuca is located in southern Arizona (Figure 1) and contains a forested wetland system that supports populations of the Arizona treefrog (*Hyla wrightorum*), which is under consideration as a candidate for listing under the ESA. This species is found in ephemeral and permanent aquatic habitats, including ephemeral streams, cienegas, and the marshy margins of ponds, where they are threatened by native and non-native predators as well as potentially the amphibian pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*, Bd). Fort Huachuca also supports potential habitat for the northern Mexican gartersnake (*Thamnophis eques megalops*), a federal candidate for listing. This species occurs in streams, cienegas, and ponds

(USFWS 2011) and is threatened by competition and predation by nonnative species, including the American bullfrog (*Lithobates catesbeianus*) (USFWS 2011). Traditional survey methods for northern Mexican gartersnakes have had low detection rates (Mixan 2009).

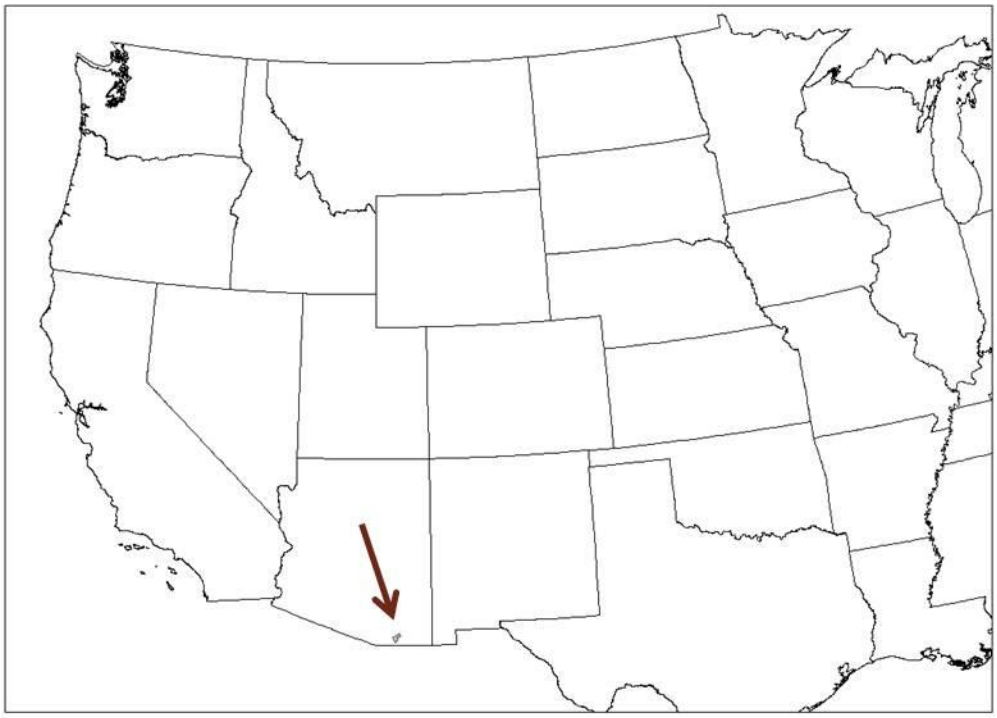

Figure 1. Location of Ft. Huachuca, Arizona.

We developed and applied eDNA tests for these two at-risk species and their invasive threats in and around Fort Huachuca, Arizona. For comparison, we also conducted field surveys or worked concurrently with Arizona Game and Fish Department field biologists conducting field sampling for target species. We used occupancy modeling techniques to investigate covariates of detection probabilities to better understand how sampling using eDNA can be most efficiently conducted. We also applied habitat modeling to a combined eDNA and field dataset for the Arizona treefrog to create a predictive map of potential habitat to guide surveys for additional populations of this species. This report is the product of year 1 of a planned two year study.

2. METHODS

2.1. Field collection

We conducted concurrent field and eDNA surveys for target species to create comparative datasets. For Arizona treefrogs, we conducted dipnet surveys at 15 sites in and around Fort Huachuca August 18-26, 2012 (Figure 2). We targeted locations that a previous survey (Vernadero Group, Inc. 2012) had identified as good or marginal habitat for Arizona treefrogs as well as some historic sites. We tested samples from these sites for Arizona treefrogs, Bd, northern Mexican gartersnakes, and American bullfrogs. For northern Mexican gartersnakes, we also worked with trapping and visual surveys conducted by the Arizona Game and Fish Department along the lower Santa Cruz River and in the San Rafael Valley, Arizona (Figure 3). We tested samples from these sites for northern Mexican gartersnakes and American bullfrogs.

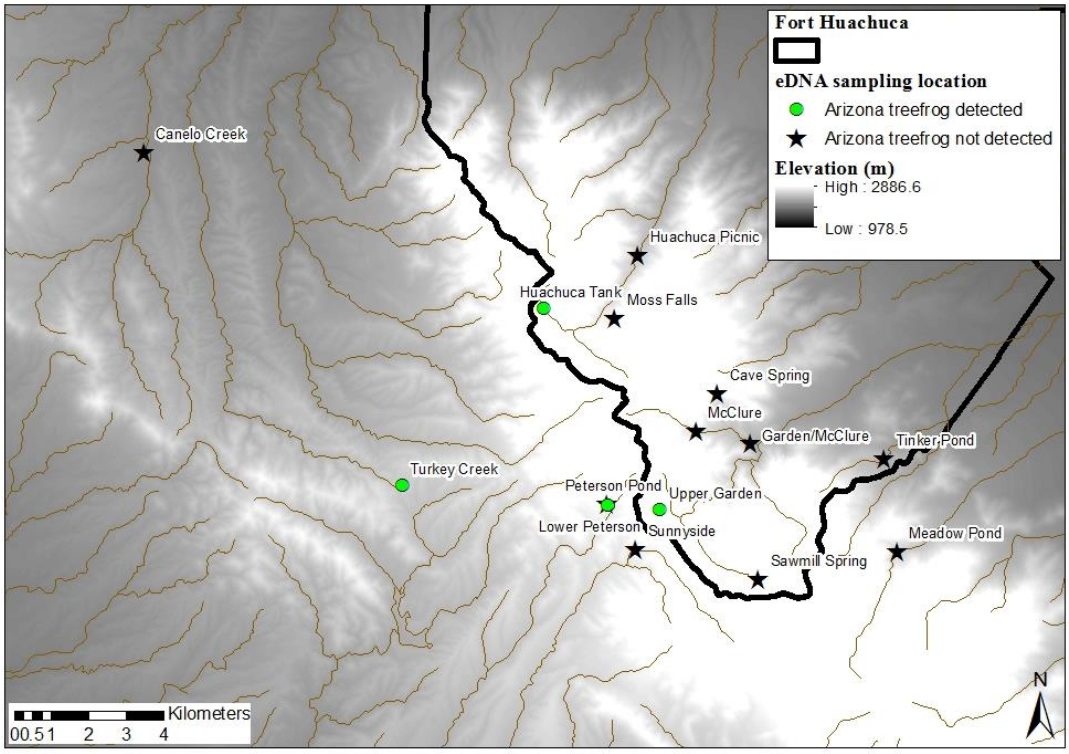

Figure 2. Occupancy results from eDNA sampling for Arizona treefrogs (*Hyla wrightorum*) at sites on and near Fort Huachuca, Arizona. Out of 15 sites sampled, Arizona treefrog DNA was detected at 4 sites (green circles) and was not detected at 11 sites (black stars).

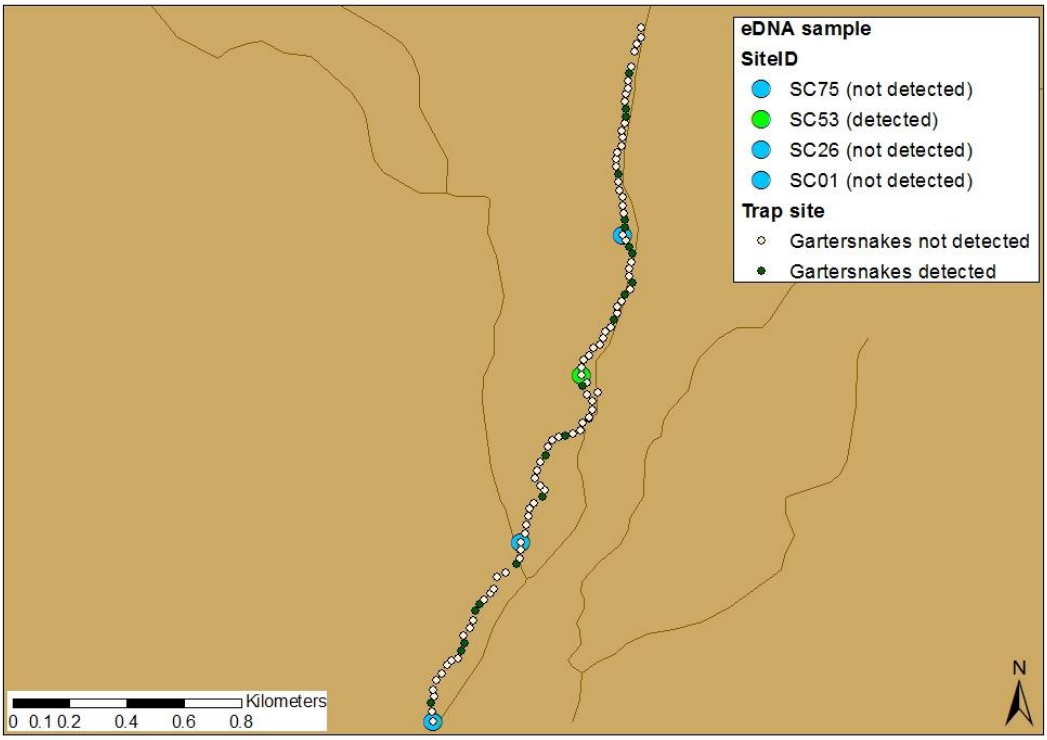

Figure 3. Survey results for northern Mexican gartersnakes (*Thamnophis eques megalops*) from eDNA sampling and trapping efforts along the Santa Cruz River (trapping conducted by Arizona Game and Fish Department), August 27-30, 2012.

At each sampling site, we collected four eDNA samples and one negative control sample (distilled water) using 47 mm diameter 0.45 µm pore size cellulose nitrate filters in disposable filter funnels (Whatman) from a single location within the site. We chose the sunniest shallow area at the wetland to sample because such areas may be more favorable to growing tadpoles (and the gartersnakes that eat them). For northern Mexican gartersnakes along the Santa Cruz River, access to water was difficult in places and samples were taken from shore where the sample crew could reach. For most sampling sites, we filtered the water on site. However, due to coordination with field crews and security issues at the U.S./Mexico border, in the San Rafael Valley we collected water samples in polypropylene bottles, put them on ice in a cooler, and filtered them within 24 hours. This method has been shown to produce equivalent amounts of eDNA to filtering on-site (Pilliod et al. 2013). We preserved filters in 95% ethanol. For lentic sites, we filtered up to 250 mL, or less if the filter clogged before the full amount was processed. Along the Santa Cruz River (a cienega at the time of sampling), we filtered 500 mL. At each eDNA sampling site, we also measured canopy cover, water temperature, pH, and conductivity. For lentic sites, we measured area by mapping using a Trimble XT GPS. We collected tissue

samples for up to 5 of each closely-related non-target species and 10 of each target species from field sites or other researchers for validation of species-specific assays (Appendix A).

2.2. Laboratory analysis

We designed quantitative PCR (qPCR) tests for the three vertebrate species. These tests use species-specific sequences to generate a positive result only when DNA of the target species is present and produce a negative result for DNA of all other species. We designed these tests from previously published mitochondrial sequences (Wood et al. 2011, D. Wood pers. comm., Gergus et al. 2004 (via T. Reeder), Austin et al. 2004) and tested them with target species DNA as well as DNA from all closely-related species in the study area (Table 1). For Bd, we used a globally standardized test (Boyle et al 2004, Hyatt et al. 2007). Reactions were 15 µl in volume and each included 3 µl of sample. Reagents in the reaction were Quantitect Multiplex PCR Mix (Qiagen, Inc.) with recommended multiplexing concentrations (1X QuantiTect Multiplex PCR mix, 0.4 mM of each primer, and 0.4 mM of each probe). We used an Applied Biosystems 7500 Fast Real-Time PCR System to run the qPCR thermal cycling (15 min at 95°C followed by 50 cycles of 94°C for 60 s and 60°C for 60 s, repeated for 50 cycles) and display the results of each reaction. The Arizona treefrog and Bd tests were run together while the bullfrog and northern Mexican gartersnake tests were each run individually.

Table 1. Quantitative PCR assays developed for target species.

Species	Primer/Probe name	Sequence
Arizona Treefrog	HYWRF	CGCTCCATTCCAAATAAGCTAGGA
	HYWRR	AGGCGGTGGTTCGTTGGTTAG
	HYWRProbe	CAL Fluor Red 610-AGTCCTCGCCCTCCTCTTCTCCAT-BHQ2
American Bullfrog	BullfrogF	TTTTCACTTCATCCTCCCGTTT
	BullfrogR	GGGTTGGATGAGCCAGTTTG
	Bullfrog Probe	NED- TTATCGCAGCAGCAAGT -MGB
Northern Mexican Gartersnake	THEQ2F	CGAAAAGGCCCTAACCTGG
	THEQ2R	CTATGATTGGTGAYAGGGTGAACAGT
	THEQ2Probe	6FAM- AATAGGCCTTCTACAGCCTA-MGB

We extracted filter samples using the Qiashredder/DNeasy method described in Goldberg et al. (2011). All filter sample extractions and qPCR set up was conducted in a lab dedicated to low-quantity DNA samples. Researchers are required to shower and change clothes before entering this room after being in a high-quality DNA or post-PCR laboratory, and no amphibian or reptile tissue samples have ever been handled in this room. A negative extraction control was included with each set of filter extractions and an additional negative qPCR control (water) was run with each plate of samples. We used a multi-tube approach for analysis, where multiple reactions were conducted for each sample, to increase the probability of detecting each species in each sample (Taberlet et al. 1999). We analyzed each sample in triplicate and included an internal positive control (IC; Qiagen) in each well. A positive sample was defined as any sample that showed exponential amplification in all three wells the first time it was tested or in one or more wells from two separate reactions (samples were rerun whenever the original triplicate wells yielded inconsistent results). Samples that showed inhibition (lack or delay of IC amplification) were rerun at 0.1X. For the vertebrates, quantitative standards consisted of diluted DNA extracted from skin tissue samples. These tissue sample extracts were quantified on a Nanodrop spectrophotometer and diluted 10^{-3} through 10^{-6}. For Bd, standards were provided by the Australian Animal Health Laboratory (Geelong, Victoria, Australia).

2.3. Data analysis

To optimize detection of target species, it is critical to know the factors that affect the probability of detecting the species in eDNA samples. Using our environmental covariates (canopy cover, water temperature, pH, and conductivity, and pond area) as well as the volume of water filtered per sample and the number of replicate samples, we used an occupancy modeling approach to analyze the covariates' effects on the probability of detecting each target species. Occupancy modeling (Mackenzie et al. 2006) uses a series of detections and non-detections to model the probability of the species occurring at a site. After building a set of occupancy models with different combinations of the covariates described above, the model that is best supported by the data is determined using an information theoretic approach (Burnham and Anderson 2002). We had planned to use this approach to model detection for each of the four species, but there was not enough variation in detection rates for Arizona treefrogs and Bd (both detected at 100% in samples from this study) to this approach. We therefore conducted occupancy modeling only for northern Mexican gartersnakes and American bullfrogs. However,

we did calculate overall detection probability for Bd based on 45 sites sampled in southern Arizona (5 in both years: 2012 and 2013) during this and a concurrent project; we could not conduct standard occupancy modeling because the number of samples (and thus the number of occasions in the occupancy modeling analysis) differed among projects. For American bullfrogs, we also combined the data from this project with that of a concurrent project in southern Arizona to create a larger dataset. For American bullfrogs and northern Mexican gartersnakes, we tested for the influence of measured environmental covariates in explaining variation in detection probability at sites with evidence of occupancy (psi = 1) using program PRESENCE (Mackenzie et al. 2006).

To create a predictive model for potential habitat of Arizona treefrogs, we used logistic regression in program R 2.13.0 (R Development Core Team 2011). Because the dataset for this project was small (15 sites), we added historical locations from the sampling area obtained from the Arizona Game and Fish Heritage Data Management, yielding a total of 22 locations evenly balanced between occupied sites and those where Arizona treefrogs were undetected. We used a set of uncorrelated ecologically-relevant climate variables (maximum temperature in the warmest month, mean summer precipitation) and a topographic variable associated with moisture (Compound Topographic Index; Geomorphometry and Gradient Metrics (version a1.0), J. Evans) to form candidate models and assessed support for them using AICc. Climate data were collected using ClimateWNA (Wang et al. 2012) with a cell size of 30 m, using data from 1981-2010. We then applied this model to the landscape to produce a predictive map of probability of habitat for Arizona treefrogs in pine-oak woodlands.

3. RESULTS

All new tests (Arizona treefrog, northern Mexican gartersnake, and American bullfrog) passed validation, testing positive only for the target species and negative for all other species. We detected Arizona treefrogs at four sites using eDNA with 100% detection probability (4/4 samples testing positive; Figure 2) as well as with dipnet and visual surveys. Because of this lack of variation in detection probability, we could not model what factors affected detection as we could with American bullfrogs and northern Mexican gartersnakes. Model evaluation indicated that the most likely model explaining the presence of Arizona treefrogs during the breeding season included maximum temperature in the warmest month and mean summer precipitation (Table 2; $X^2 = 13.32$, $P = 0.0013$, pseudo-$R^2 = 0.61$, ROC = 0.868). Although the full model was competing with this simpler model, using the principle of parsimony we applied this climate-only model to create the predictive surface (Figure 4).

Table 2. Candidate models for predicting where populations of the Arizona treefrog (*Hyla wrightorum*) may occur. MSP = mean summer precipitation, MWMT = mean warmest month temperature, CTI = compound topographic index.

Model	AICc	ΔAIC	Weight
MSP + MWMT	24.51	0.00	0.713
Full	27.45	2.94	0.164
MWMT	29.15	4.64	0.070
MWMT + CTI	30.74	6.23	0.032
Null	32.70	8.19	0.012
MSP	34.74	10.23	0.004
CTI	35.06	10.55	0.004

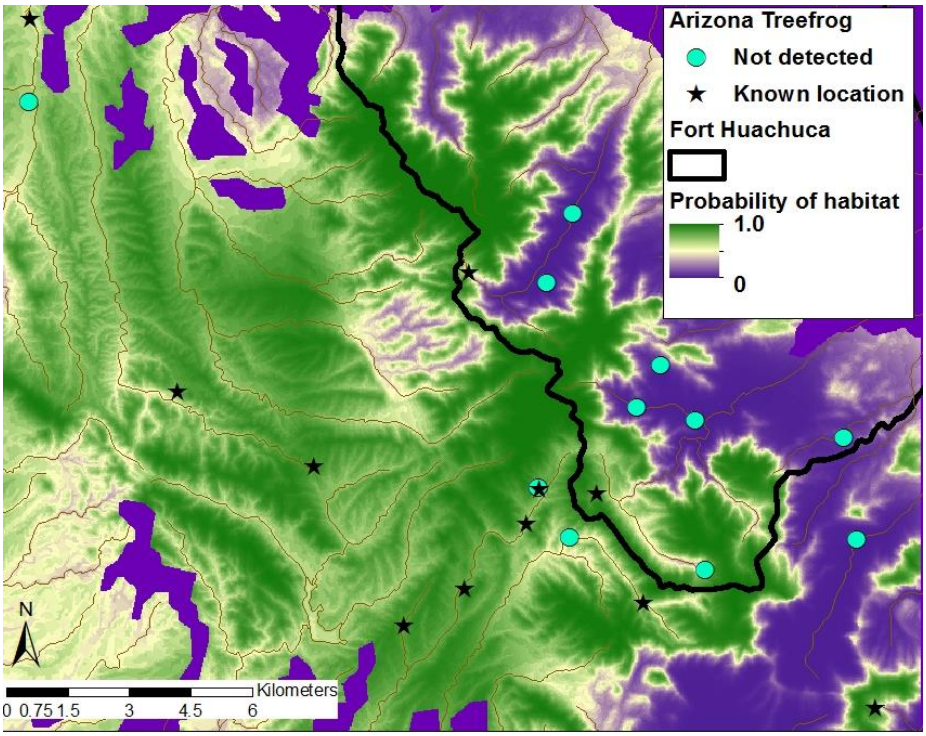

Figure 4. Known locations and predicted probability of occupancy of the at-risk Canelo/Huachuca Distinct Population Segment of the Arizona treefrog, based on environmental DNA collections in Year 1 and historical data.

We detected northern Mexican gartersnakes at two sites using eDNA (Figure 5). Both of these sites are known locations for this species. However, we did not detect this species using eDNA at three of the sites along the Santa Cruz River or Pasture 9 tank, where they were detected using visual surveys and trapping concurrent with our sampling. At the Santa Cruz River, eDNA samples were collected prior to trapping so that trapping efforts would not introduce extraneous eDNA to sampling sites. Because we did not know exactly where snakes would be caught, this resulted in eDNA sampling not directly overlapping with locations where snakes were trapped (Figure 4), and pools along the Santa Cruz River at the time of sampling were not necessarily hydrologically connected at the time of sampling. Occupancy modeling for northern Mexican gartersnakes indicated that conductivity was the most likely explanation for differences in detection probabilities between sites, although sample size was very small (Table 3). Increases in conductivity were found to be related to increased detection probability, with estimates ranging from probabilities of 0 (where conductivity = 227) to 0.75 (where conductivity = 436) (Figure 6). We did not conduct predictive modeling for this species because the number

of known locations (including this study and records from Arizona Game and Fish Department) was too small (N = 11 records of presence in the area) for accurate modeling.

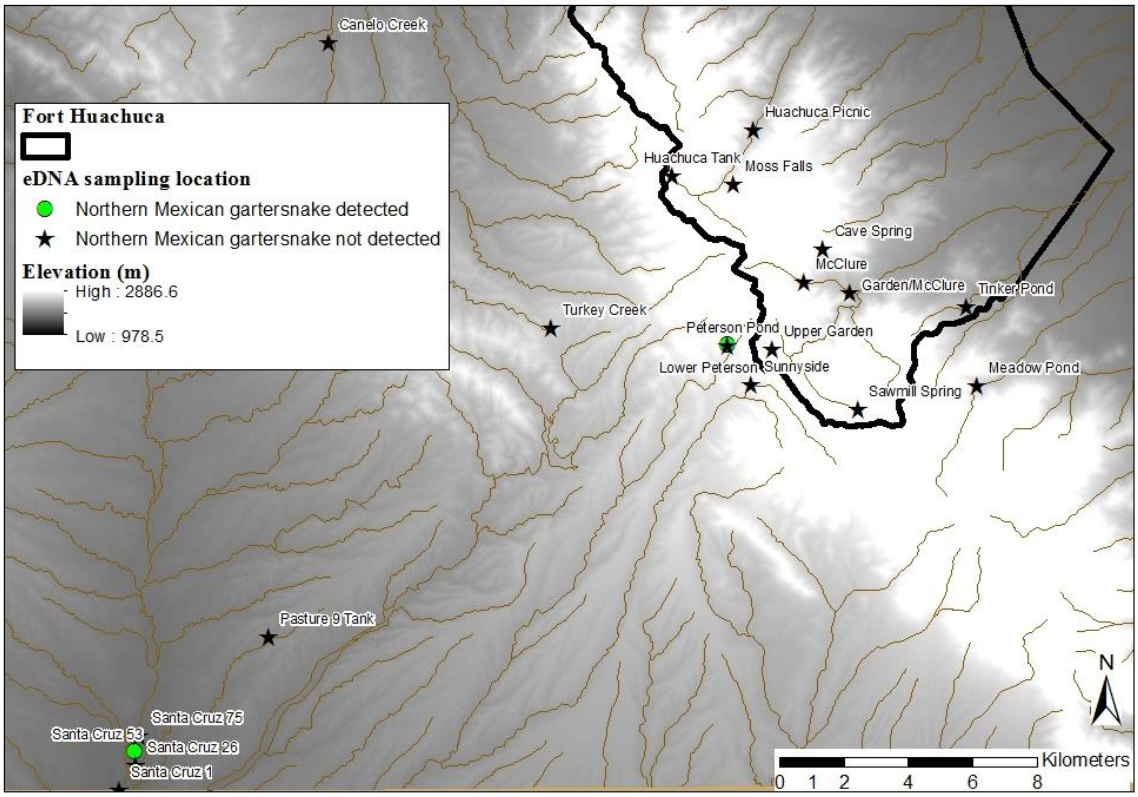

Figure 5. Occupancy results from eDNA sampling for northern Mexican gartersnakes (*Thamnophis eques megalops*) at sites on and near Fort Huachuca, Arizona. Out of 20 sites sampled, northern Mexican gartersnake DNA was detected at 2 sites (green circles) and was not detected at 18 sites (black stars).

Table 3. Models of northern Mexican gartersnake detection probability at sites with known presence (psi = 1).

Model	AIC	ΔAIC	Weight
Conductivity	15.30	0	0.911
Temperature	22.17	6.87	0.029
pH	22.19	6.89	0.029
Null	23.63	8.33	0.014
Canopy cover	24.86	9.56	0.008
Sample volume	25.47	10.17	0.006
Sampling replicate	26.45	11.15	0.004

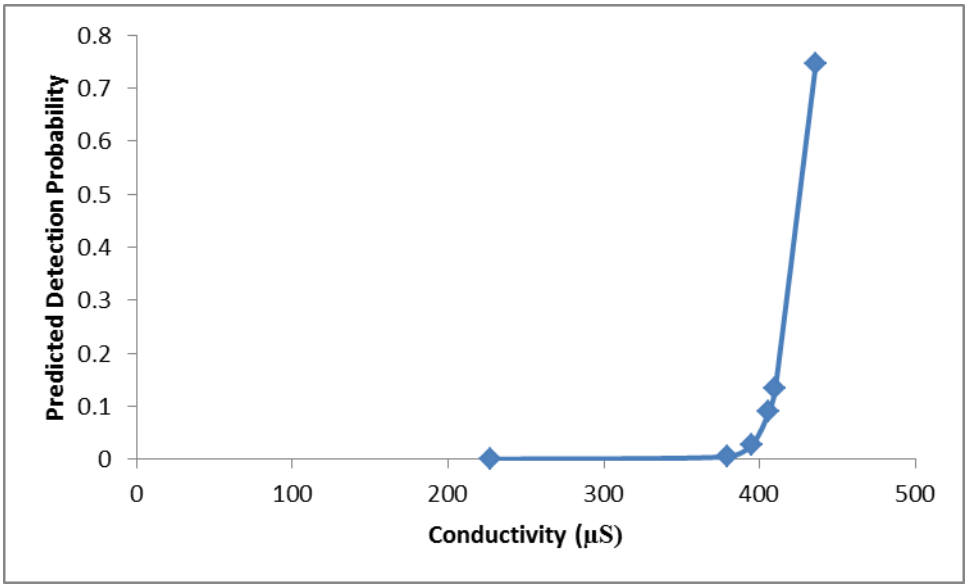

Figure 6. Predicted relationship of conductivity and eDNA detection probability for the northern Mexican gartersnake. This result is based on few data points, however, and should be further investigated.

We detected American bullfrogs at five sites sampled for this project; four of those were sites where field crews also detected this invasive species (bullfrogs were not detected by field crews at Pasture 9 tank; Figure 7). In the combined dataset, this species was detected at 15 sites by field crews and 19 sites using eDNA, with one site detected by field crews that was missed by eDNA and four sites that tested positive for eDNA where field crews did not detect the species. For occupancy modeling, we excluded the four sites along the Santa Cruz River because their areas were not estimated, leaving 16 sites for modeling. The most likely explanation for variation in eDNA detection probability for American bullfrogs was temperature (Table 4), with all sites where eDNA detected bullfrogs in one or fewer samples being ≥25°C water temperature. Canopy cover was excluded from this analysis for lack of variation (all sites < 10%). Detection probabilities are predicted to decline for this species as temperature increases (Figure 8); however, there was also some evidence for the null model (AIC weight = 0.08), indicating a larger sample size is necessary to determine if this is an important factor. We did not create a predictive model of presence for this species because the goal of the study was to use eDNA to create predictive maps of the at-risk species in this study (Arizona treefrogs and northern Mexican gartersnakes) for finding additional populations; American bullfrogs were tested for to determine the co-occurrence of this threat with target species and demonstrate that eDNA can be used to simultaneously detect threats and target species that co-occur.

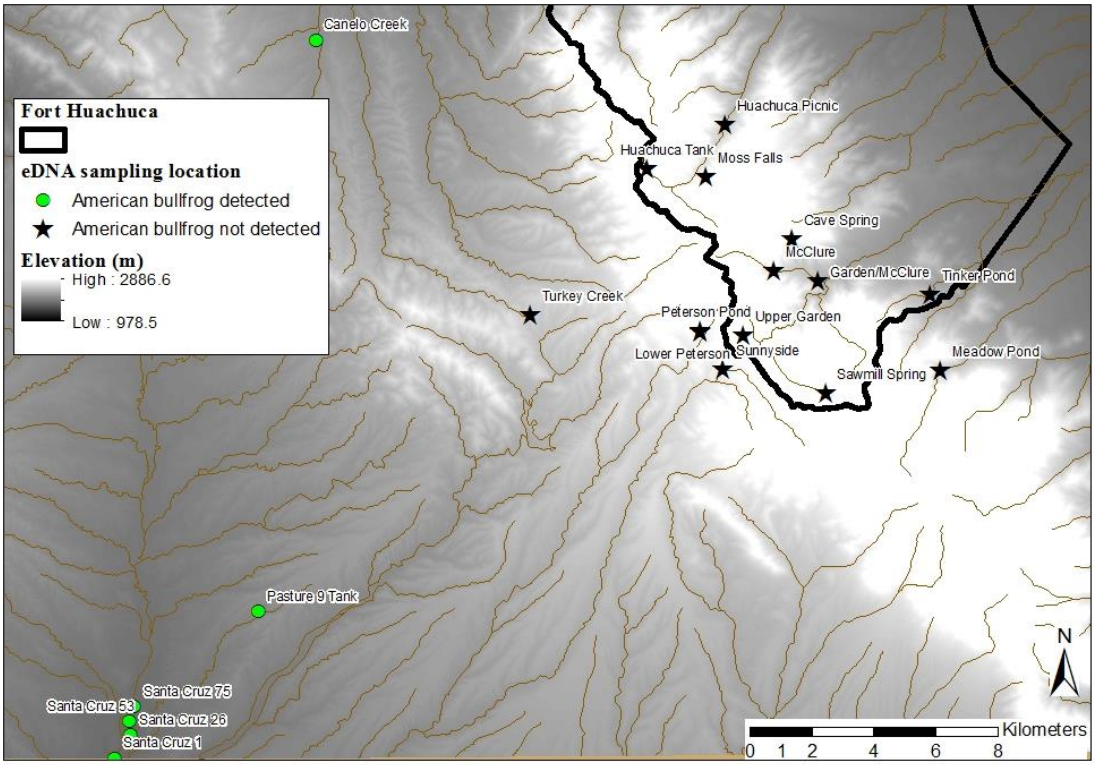

Figure 7. Occupancy results from eDNA sampling for American bullfrogs (*Lithobates catesbeianus*) at sites on and near Fort Huachuca, Arizona. Out of 20 sites sampled, American bullfrog DNA was detected at 6 sites (green circles) and was not detected at 14 sites (black stars).

Table 4. Models of American bullfrog detection probability at sites with known presence (psi = 1).

Model	AIC	ΔAIC	Weight
Temperature	74.28	0	0.531
Null	78.05	3.77	0.081
Conductivity	78.78	4.50	0.056
Area	79.37	5.09	0.042
pH	79.95	5.67	0.031
Sample volume	80.02	5.74	0.030
Sampling replicate	83.74	9.46	0.005

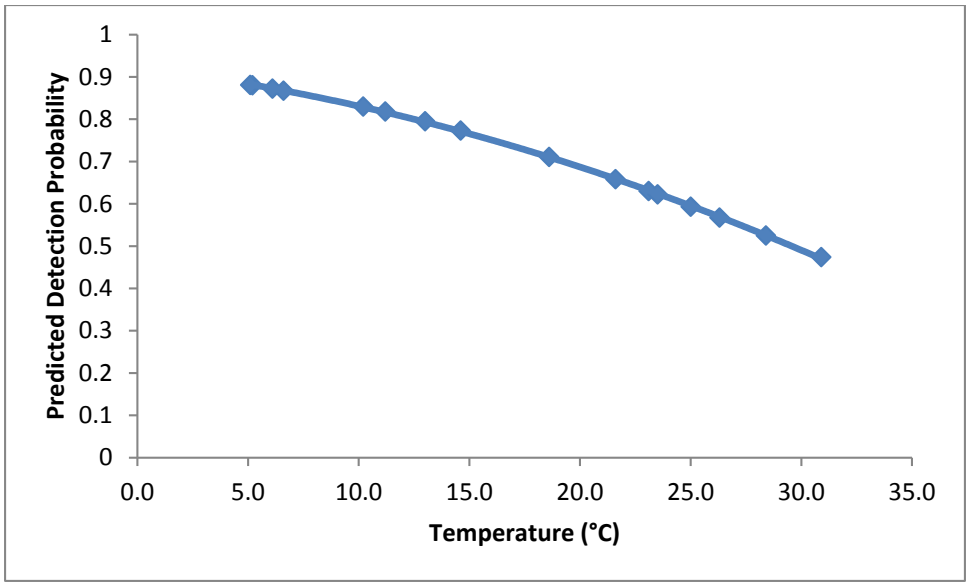

Figure 8. Predicted detection probabilities for American bullfrogs using eDNA sampling as related to water temperature.

We detected Bd using eDNA from one site during this study with 100% detection probability (4/4 samples testing positive; Figure 9). As with the Arizona treefrog, because of lack of variation in detection probability, we could not model factors influencing detection. In the combined dataset of 50 site visits to 45 sites of interest to amphibian management in southern Arizona, we detected Bd using eDNA at seven sites with an average detection probability of 0.80 (95% CI: 0.59 – 1.00). As with the American bullfrog, we did not create a predictive model of presence for this species because the goal of the study was to use eDNA to create predictive maps of the at-risk species in this study.

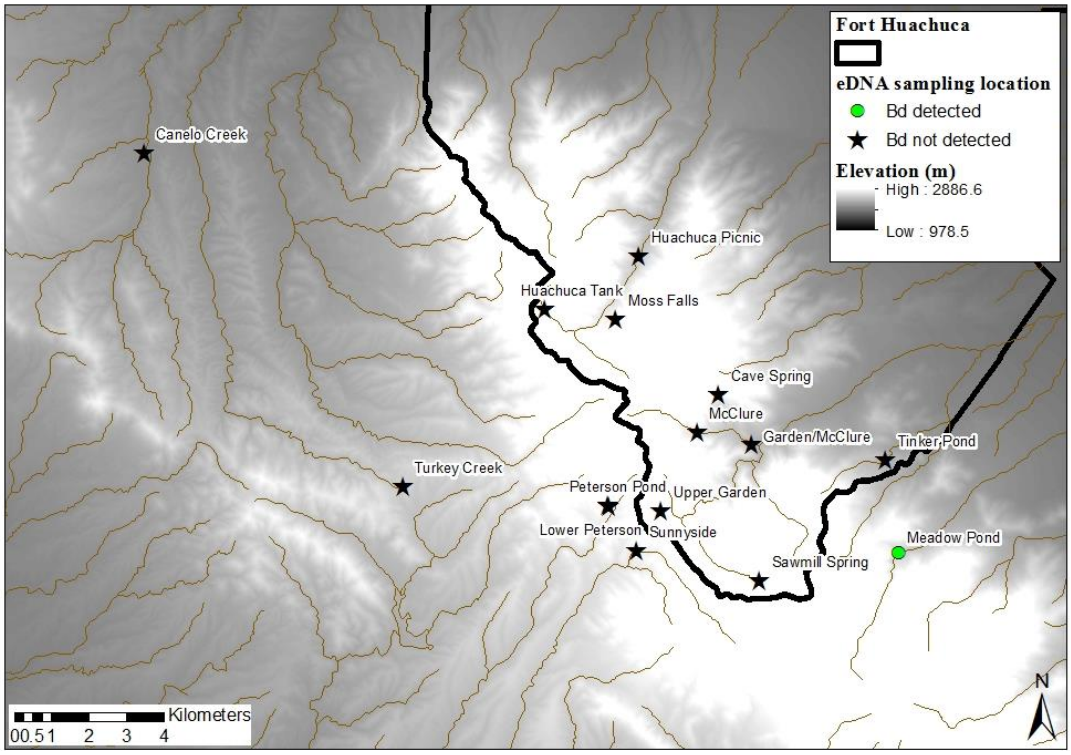

Figure 9. Occupancy results from eDNA sampling for the amphibian pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*; Bd) at sites on and near Fort Huachuca, Arizona. Out of 15 sites sampled, Bd DNA was detected at 1 sites (green circles) and was not detected at 14 sites (black stars).

All negative controls from field sites, extractions, and qPCR reactions tested negative.

4. DISCUSSION

4.1. Detection probabilities

Estimated detection probabilities were high for Arizona treefrogs and Bd, moderate for American bullfrogs, and low for northern Mexican gartersnakes. This range of variation indicates that more work needs to be done to understand covariates of detection probabilities for different species and inform sampling design. Important components of sampling design for eDNA work include number of samples taken at a site, volume sampled, and number and characteristics of locations sampled within a site.

For Arizona treefrogs, eDNA was a very effective tool for detection, but visual and dipnet surveys provided the same information. Our surveys were conducted later in the season than ideal (due to delayed funds) and we may have missed detecting the species at sites where breeding did not succeed or was complete before our arrival. For species that can be highly detectable at some sites or when population sizes are large, a hybrid eDNA and field approach is likely the most cost-effective. For the Arizona treefrog, this would start by the field crew conducting a visual survey (without entering the water) at a wetland. If no individuals of the target species were detected, water samples would be collected and temporarily stored (for example, in whirl-paks in the shade) while dipnetting took place. This is to ensure that extraneous DNA from dipnets and boots does not enter the water (disinfecting boots and nets for DNA requires 50% household bleach, more than is typically used for decontamination). If the species was not detected using dipnets, then the water would be filtered and filters stored for eDNA analysis. In this way, the benefits of the high probability of detection of eDNA are incurred without unnecessary costs for collecting and analyzing samples at sites where the species is easily detectable using less expensive techniques.

The map produced from the model of Arizona treefrog locations based on climate data indicates additional areas that could be surveyed for this species on and around Fort Huachuca. The discovery of additional populations of Arizona treefrogs could reduce potential restrictions on land use on the base by indicating that the Huachuca/Canelo Hills Distinct Population Segment is larger than is currently known. The importance of the mean temperature in the warmest month to the potential habitat of the Arizona treefrog indicates that warming temperatures may reduce habitat for this species in the future, making thorough mapping of populations of this species critical for predicting future threats. There are also fine-scale

processes affecting the distribution of this species that are not included in this climate-based map. For example, the two ponds at Peterson Ranch have different amphibian assemblages. The shallower ephemeral pond is habitat for Arizona treefrogs, while the neighboring deeper pond contains tiger salamanders and Chiricahua leopard frogs (although the latter also attempt breeding in the ephemeral pond). Environmental DNA results indicate that there is little if any use of the permanent pond by Arizona treefrogs (at least during the period sampled). Additional surveys to improve this model will help managers better understand the status and potential threats to this species at local and regional scales. One benefit of eDNA sampling is that many species can be tested for using the same filter, which can provide important information about predator and prey communities of target species.

Detection probabilities for the northern Mexican gartersnake were low, probably due to reduced shedding of skin cells from the more closed skin of this reptile as compared with amphibians. This demonstrates that detection of this aquatic reptile is possible using eDNA, but the protocol needs to be improved to be more sensitive to this species before it is useful for applied management. While we did not sample directly from areas where the snakes were trapped along the Santa Cruz River, this is the highest-density population known for this species. To be cost-effective, the eDNA test needs to be able to detect the species throughout the drainage, as snakes are likely using most of the area. There was some evidence from this very small dataset that conductivity may increase the probability of detection of eDNA, a pattern that we have not seen for other species. A process that would explain this pattern would be the binding of eDNA to inorganic dissolved solids. However, because this pattern was found in such a small dataset, this result should be taken with caution until additional data can be collected.

One of the benefits of the eDNA approach is that pathogens can be detected using the same filter sample as at-risk species. In this area, we found high detection probabilities for Bd, given that it was detected in the water. We are following up on this research by conducting paired tests of amphibian swabs and water samples at several sites to have an external measure to compare with this detection probability. Bd sampling from water is currently generally accomplished using a polyethersulfone filter that has fairly low probabilities of detection (Hyman and Collins 2012). The cellulose nitrate filters we applied have been shown in the past to detect Bd (Walker et al. 2007) and this study indicates that this method may compare favorably to the one currently in use.

The final species that we detected using eDNA was the invasive American bullfrog. This species is a threat to native amphibians throughout the western United States (Casper and Hendricks 2005) and although it can be highly detectable using auditory and visual sampling, we detected the species using eDNA at 20% more sites than field crews. This large improvement in detection of this invasive species using eDNA over field surveys has also been shown in European populations (Dejean et al. 2012). The exception to this was one site that had a dense population of bullfrogs where we did not detect the species using eDNA, which models indicate may have been due to the high temperature at the site. These results provide additional support for a hybrid approach of applying both visual and eDNA surveys to detect amphibian species. The decrease in detection probability of eDNA with higher temperatures may indicate that eDNA degradation, which occurs faster at higher temperatures (Strickler et al., in prep), may be a limiting factor in the detection of species using eDNA in warm climates. However, the weight of evidence for the null model in this set also indicates that more data collection is necessary before conclusions can be drawn.

4.2. Cost efficiency

The cost efficiency of eDNA over field collection lies in the increased probability of detection of eDNA techniques. Costs of using eDNA versus traditional field sampling techniques are approximately equal when the detection probability for one eDNA sample is equal to the detection probability for one hour of a crew's field work (from a project biologist and a technician together; Figure 10A). However, detection probabilities of eDNA have been shown to be substantially higher than field surveys (Jerde et al. 2011, Dejean et al. 2012). In this case, cost savings result when higher probabilities of detection at a site can quickly be realized using multiple eDNA samples in a single short visit, compared with multiple or longer visits needed by field crews (Figure 10B). These graphs can serve as approximate guidelines for determining when eDNA sampling will have the most benefit. Multiple species can also be tested for with a single eDNA sample, reducing costs of eDNA analysis below those estimates. Costs will also be dependent on the charge for sample processing from commercial or service laboratories. The global community of eDNA researchers is currently preparing a best practices paper to address issues of laboratory quality control and assurance (Goldberg et al., in prep). Guidelines for assessing a laboratory for eDNA sample analysis are listed in Appendix B.

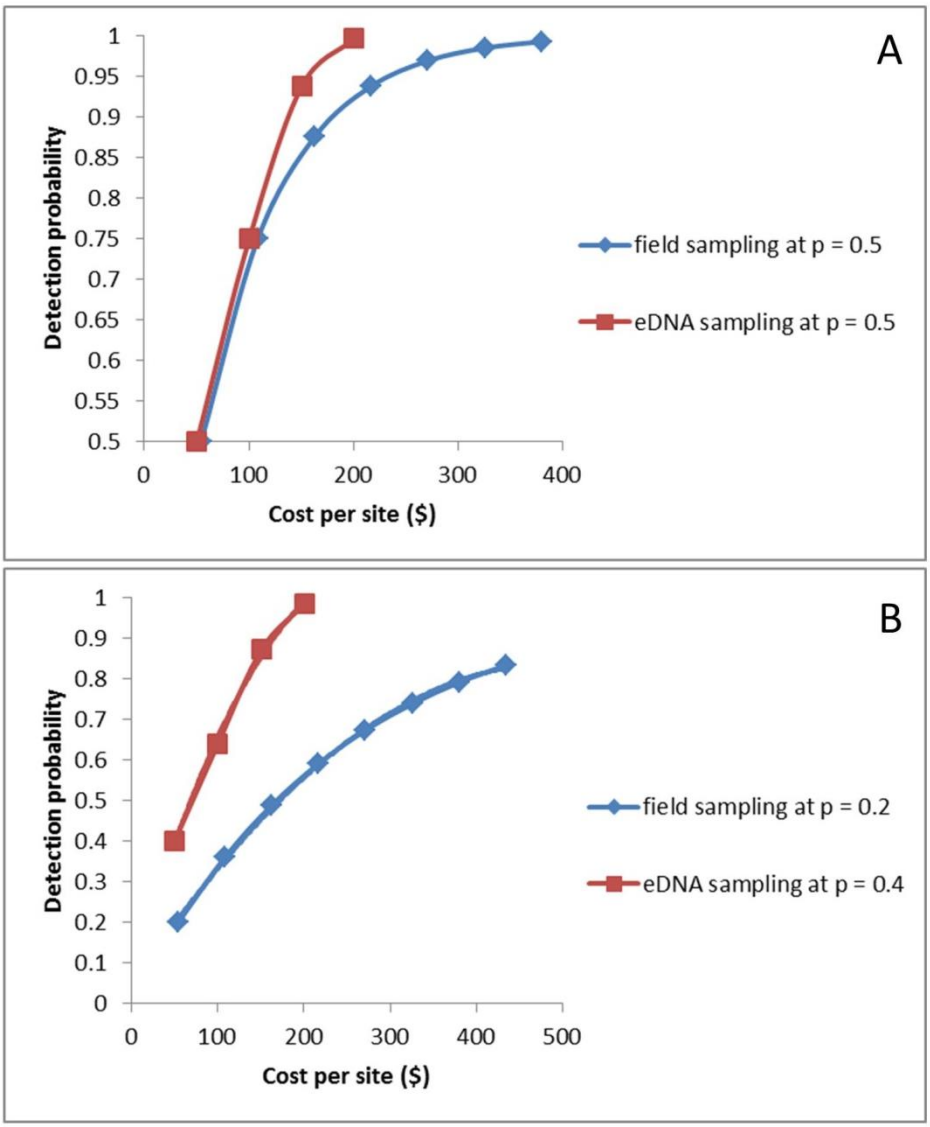

Figure 10. Detection probabilities predicted by costs per site for sampling when A) Detection probabilities for eDNA and traditional field sampling are equal for one eDNA sample and one hour of field crew sampling and B) detection probability is greater for eDNA. Costs are calculated assuming one eDNA sample takes 15 minutes of a technician's field time at $20.77/hour, plus the cost of the filter and filter analysis and one hour of a crew's field work consists of a project biologist (at $33.50/hour) and a technician together. Time for traveling between sites and costs of start-up (assay development, field equipment, training, and permitting) are not included.

5. CONCLUSIONS

We have demonstrated the high potential for eDNA to contribute to the detection and monitoring of amphibian and reptile species on Department of Defense lands and beyond. Methods for detecting amphibians and the amphibian pathogen Bd have been successful and are ready for further application, including validating and improving on the model of potential habitat developed in this study for Arizona treefrogs. Methods for detecting aquatic reptiles need further development, including testing different filter types that could filter more water before becoming clogged or improvements in DNA extraction and processing methods from filter samples.

From this first year of this project, we can conclude that eDNA detection probabilities can be very high but also vary among species. General protocols will need to be modified for specific species and systems to maximize species detections. Along with the field protocol we have developed for this project, we provide the following general guidelines for implementing eDNA techniques (note: many of these recommendations are based on preliminary data from a variety of projects):

1. **Determine the most appropriate season to conduct eDNA surveys.** This should be the season with the highest species density in the water, as long as that coincides with the life stage of interest. For amphibians, this is likely to be during tadpole development if reproducing populations are the target of the surveys (as opposed to adults that may not be successfully reproducing). In stream systems, low flow may be the best time to sample if the species is present year-round. If water temperatures exceed 23°C at low water, however, DNA degradation may be a limiting factor and earlier sampling may provide higher detection probabilities.

2. **Consider spatial sampling design.** If species tend to be clustered and/or aquatic systems are large, sampling at multiple locations within a site may be necessary. Consider sampling at two sites on opposite sides of wetlands for sites with area >1200 m^2 and three sites for wetlands with areas >2300 m^2. For lakes, consider sampling every 50 m around the perimeter. Samples from multiple locations at a site can be combined before filtering to reduce the number of filters needed. If it is known that species use particular microhabitats, create the sampling design around those areas. For streams, there is some evidence that slower water may accumulate more

eDNA; however, sampling from the thalweg has yielded very high detection probabilities for stream amphibians (Pilliod et al. 2013).

3. **Consider filter type.** Cellulose nitrate filters with 0.45 µm pore size have provided excellent results for many species but may clog in some systems, limiting the volume of water that can be sampled. Glass fiber filters were originally used for eDNA collection (Jerde et al. 2011), but are not as efficient as other filter types (6X more eDNA is captured in the same volume of cellulose nitrate filters, CSG, unpublished data). Other filter types in use include polycarbonate and polyethersulfone. We have shown some success with cellulose filter paper and are testing this material more broadly for systems with high levels of particulates.

4. **Consider preservation method.** We have been using ethanol to preserve filters, but drying filters may reduce transport restrictions as well as improve detection (preliminary data indicates detection of Bd in particular may be higher with this preservation technique). This can be accomplished using silica beads, but care must be taken to prevent cross-contamination of samples.

5. **Conduct a pilot study.** A pilot study is necessary to determine if a proposed sampling design will yield high enough detection probabilities to be useful and cost-efficient. Collecting covariates of detection (area, canopy cover, water temperature, conductivity, stream discharge, volume sampled, etc.) can yield insights to improve sampling design if detection probabilities are not optimal.

6. **Consider how eDNA sampling can complement existing field methods.** Some field methods yield high detection probabilities with low effort under certain conditions. Environmental DNA benefits are highest when species are difficult to detect with standard methods. For some species, eDNA surveys may be best applied on a site-by-site basis when standard surveys do not detect the target species. Because of contamination issues, if boots, nets, or other materials will be in the water before it is determined if eDNA samples will be taken, water should first be collected and stored in the shade during the survey and then discarded or filtered depending on the outcome of the survey.

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APPENDIX A.

TARGET AND NON-TARGET SPECIES VALIDATED FOR EDNA TESTS

Target and non-target species validated for eDNA tests.

Species	Number Validated
Anurans	
American bullfrog (*Lithobates [Rana] catesbeianus*)	10
Arizona treefrog (*Hyla wrightorum)*	10
Barking frog (*Craugaster augusti*)	5
Canyon treefrog (*Hyla arenicolor*)	5
Chiricahua leopard frogs (*Lithobates [Rana] chiricahuensis*)	5
Couch's spadefoot (*Scaphiopus couchi*)	5
Great Plains toad (*Anaxyrus [Bufo] cognatus*)	5
Lowland leopard frog (*Rana yavapaiensis*)	5
Mexican spadefoot (*Spea multiplicata*)	5
Red-spotted toad (*Anaxyrus [Bufo] punctatus*)	5
Sonoran Desert toad (*Incilius [Bufo] alvarius*)	5
Tarahumara frogs (*Lithobates [Rana] tarahumarae*)	5
Western narrow-mouthed toad (*Gastrophryne olivacea*)	5
Woodhouse's toad (*Anaxyrus [Bufo] woodhousii)*	5
Gartersnakes	
Northern Mexican gartersnake (*Thamnophis eques megalops*)	10
Checkered gartersnake (*Thamnophis marcianus*)	1
Black-necked gartersnake (*Thamnophis cyrtopsis*)	2

APPENDIX B.

GUIDELINES FOR SELECTING A LABORATORY TO PROCESS ENVIRONMENTAL DNA SAMPLES

Guidelines for selecting a laboratory to process environmental DNA samples:

1. Environmental DNA samples are very low quality and quantity compared with DNA samples collected directly from organisms. They therefore need to be handled in a separate room (clean room) from high-quality samples and the products of polymerase chain reactions (PCR). This clean room needs to have dedicated equipment, including pipettors, centrifuges, and any other item that is needed for sample processing. Technicians should be required to shower and change clothes or go through equivalent decontamination procedures before entering this room after having been in a lab containing PCR product.
2. For assays to be specific enough to detect only target species in eDNA samples, quantitative PCR or next-generation sequencing is required. Conventional PCR tests are not adequate. Laboratories should be aware of this and have the skills and equipment to analyze samples appropriately.
3. Laboratories should have a standard practice that filter tips should be used at all times when eDNA samples are being handled.
4. Laboratories should have a standard practice that 50% household bleach should be used to clean all surfaces between uses and when any sample touches a surface in the clean room.
5. Laboratories should have a standard practice that negative controls are extracted with each batch of extractions and tested in target species assays.
6. The laboratory should be able to archive samples after processing for future analysis, if that is requested by the agency (it is reasonable to expect an additional fee for this service).
7. The agency should collect a series of samples from known positive and negative sites and send them for a blind test to the laboratory (it is reasonable to expect to pay for this service, although some commercial laboratories may waive this cost). All sites with the species should test positive and without should test negative. However, detection probabilities may not be perfect at positive sites and sometimes field crews can introduce small amounts of DNA into samples when first learning techniques (or if clean field practices are not kept to). Ideally, this testing is an iterative process that involves working with a lab to understand where errors are occurring and fix problems during a pilot phase. Laboratories should be willing to work collaboratively with the agency during this phase and produce accurate data from blind samples to the satisfaction of the agency before embarking on extensive sample processing.

APPENDIX C.

FIELD PROTOCOL FOR eDNA SAMPLE COLLECTION

eDNA PROTOCOL
SAMPLE COLLECTION

Caren Goldberg and Katherine Strickler, University of Idaho
28 February 2014

Adapted from
Protocol Version 04/12/2012 (D.S. Pilliod, R.S. Arkle, and M.B. Laramie)
USGS Snake River Field Station

MATERIALS

1. Cellulose nitrate disposable filter funnels or other field-tested, disposable filter funnels
2. Vacuum flask (1L)
3. Silicone tubing
4. Vacuum hand pump (from auto parts store)
5. Rubber stopper with hole for funnel stem
6. Latex or nitrile gloves (non-powdered)
7. Forceps (filter forceps if possible)
8. High quality screw cap 2mL tubes with 1mL 100% molecular-grade ethanol (<u>not</u> denatured)
9. Ethanol-proof laboratory pen (do not use a regular Sharpie marker)
10. 50 mL tubes of 30 mL 1) 50% bleach solution 2) distilled water in a holder (a foam drink holder [koozie] works well)
11. Polypropylene grab bottles and cooler (for off-site filtering) or whirlpaks (for on-site filtering)
12. Water, bleach, scrub brush, and tubs (for decontaminating between sites)

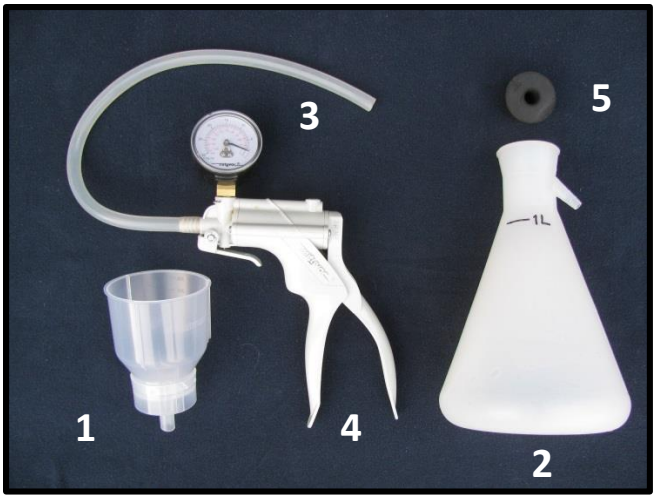

Figure 1. Filter funnel (1), vacuum flask (2), silicone tubing (3), vacuum pump (4), and rubber stopper (5).

Figure 2. Latex or nitrile gloves (6), forceps (7), 2 mL tubes with 1 mL ethanol (8), ethanol-proof lab marker (9), and 2 50 mL tubes with 50% bleach solution distilled water (10).

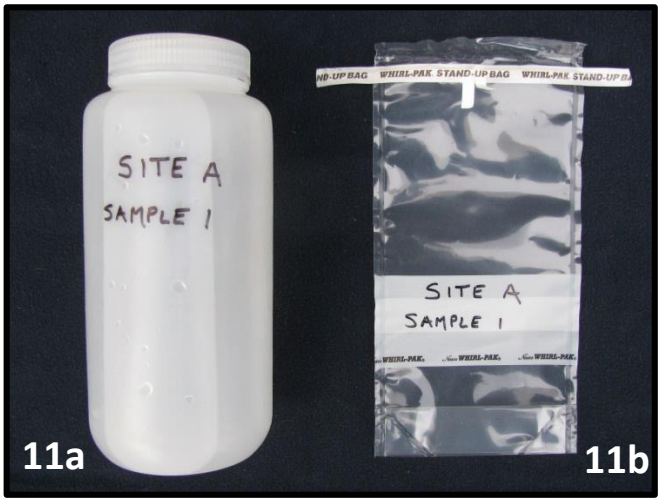

Figure 3. Polypropylene grab bottles (11a) and whirlpaks (11b).

Figure 4. Water (12a), bleach (12b), scrub brushes (12c), and tubs (12d) for decontaminating boots and equipment between sites.

CONTAMINATION PREVENTION

Avoid cross-contamination between samples! Contamination can result from a variety of factors at every step in the sample collection process. Be vigilant.

1. Wear gloves when removing filter and placing in ethanol storage tubes. Do not let gloves get contaminated before you handle the filter! Use non-powdered gloves only. Wear a glove when collecting water for sampling unless hands have been decontaminated while decontaminating boots and other gear between consecutive sites.
2. Be careful with gloves and other supplies. Do not leave them unprotected and do not toss them in a backpack. Keep everything clean and in plastic bags.
3. Open filter funnel package from bottom (stem end) and keep closed between sites.
4. When filtering samples, be careful not to touch the top or inside of the filter cup. No gloves are needed when handling the outside of the filter funnel, vacuum flask, and rubber stopper, as these are downstream from the filter (that is, they are below the filter and do not come into contact with sample water before it is filtered).
5. Decontaminate forceps in 50% bleach for at least 1 minute between each sample. Rinse well with distilled water (Figure 5).
6. Clean boots thoroughly between sites. Remove all dirt, pebbles, etc. from soles and sides of boots. Decontaminate in 10% bleach if they came in contact with water or mud during sampling. Rinse well in tap water (not water from the site) (Figure 6).
7. Bleach vacuum flask and stopper in 10% bleach between sites. Bleach pump and tubing if they got wet during sampling or filtering.
8. To re-use Nalgene grab bottles, bottles must be submerged in 50% bleach solution for at least 1 minute. Rinse thoroughly with clean water (fill, cap, shake, and rinse; repeat at least 3 times), let dry. At the sampling site, rinse again with sample water 3 times before collecting sample.
9. To test for field contamination, collect 1 field negative per site. Fill collection receptacle (whirlpak or bottle, whatever is being used for the samples) with distilled water. Using methods for filtering samples as described in Step 3 below, filter the same volume of distilled water as the volume of samples.

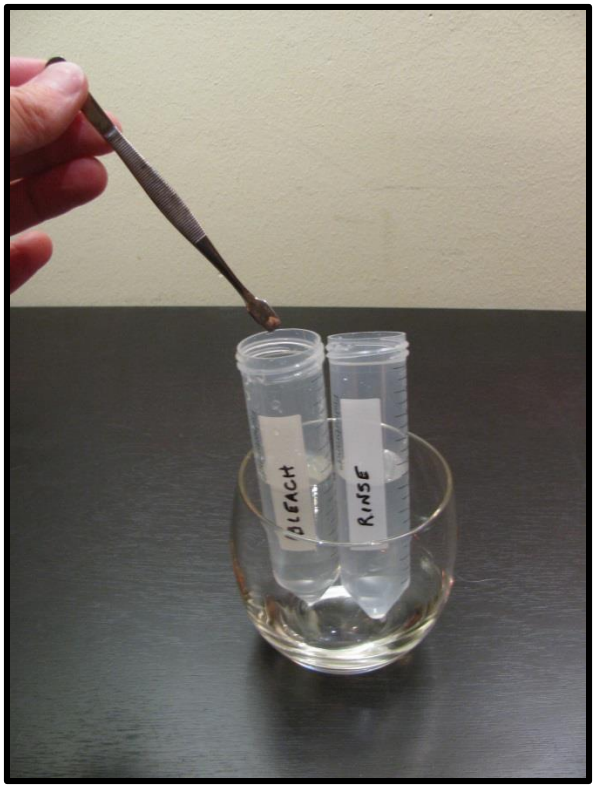

Figure 5. Decontaminate forceps in 50% bleach for at least 1 minute between each sample. Rinse well with distilled water.

Figure 6. Clean boots thoroughly between sites. Decontaminate with 10% bleach and rinse well with tap water.

SAMPLE COLLECTION

Step 1. Sample Site Selection

1. Determine criteria for selecting spot for water collection based on habitat use of the target species. Be consistent among sites.

In ponds, lakes, and wetlands, collect water at a site you predict your target species is most likely to occur. If wetland is >40 m diameter (assuming a round shape), collect additional samples from a second site 180 degrees across from first sample site. If wetland is >55 m diameter, consider using 3 sites.

In streams, many studies have collected water from the thalweg (area of strongest current, usually the deepest part of the channel) with success, but areas where the stream current is slower may contain more eDNA. This is currently being studied.

2. Take detailed notes about the sampling location within the pond, lake, wetland, or stream. Describe characteristics of the site relative to the water body as a whole. If the target species is observed before, during, or after collecting water samples, record the location of the species relative to the sampling location. Photos may also be helpful for later reference.

Step 2: Filter Assembly (Figure 7)

1. Attach rubber stopper to top of the vacuum flask.
2. Attach disposable filter funnel directly to rubber stopper by inserting stem of funnel into hole in stopper, creating airtight seal.
3. Attach vacuum pump to tube on vacuum flask using silicone tubing.

Figure 7. Filter assembly.

Step 3. Water Collection and Filtration

If filtering on-site:

1. Collect water in new whirlpak for filtering, one sample per pak.
2. Pour sample slowly into filter funnel, filling funnel to 250 mL mark (Figure 8). Pause several times to swirl water in whirlpak or bottle before pouring remaining water into funnel.
3. Engage vacuum pump to begin filtration (Figure 9). During filtering, make sure vacuum pressure is sustained (monitor pump gauge if available, or watch water level to make sure water is flowing between the funnel and vacuum flask).
4. If >250 mL is being collected, disengage vacuum pump when adding more volume if you are using the funnel to measure volume. Otherwise, use mark on flask to determine when target volume has been reached. Do not use the pressure release on the vacuum pump or water from hose may contaminate the filter sample.
5. Make note of the volume of water filtered, whether samples were collected using whirlpaks or grab bottles, and any unusual events, conditions, or problems.

If taking grab samples for later filtering off-site:

1. Collect water in sterile Nalgene bottle, one sample per bottle (Figure 10).
2. Rinse grab bottle 3 times with water from sample site. Cap and shake water during each rinse. Dispose of rinse water away from spot where you'll collect water.
3. Fill grab bottle with water away from where rinsing occurred, as much as possible while standing in one place. Avoid stirring up sediment while collecting sample.
4. Cap firmly, label with site name and sample number, and place in a cooler.
5. Filter as soon as possible (within 24 hours) using steps 2-4 described above for filtering on-site.

Figure 8. Pour sample slowly into filter funnel, filling funnel to 250 mL mark.

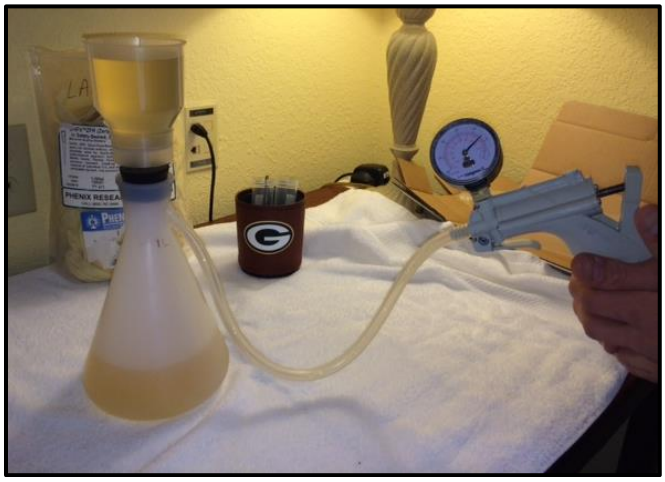

Figure 9. Engage hand pump to begin filtration.

Figure 10. Collect water in sterile Nalgene bottle.

Step 4. Filter Membrane Removal

1. Decontaminate forceps by soaking in 50% bleach solution for at least 1 minute and then in deionized or distilled water, each stored in a 50 mL tube.
2. Remove funnel cup. Pull tab on side of funnel and gently twist to disconnect top and bottom units, exposing filter membrane (Figure 11).
3. After removing top of funnel, wear clean glove (nitrile or other single-use gloves) on hand that will touch filter paper.
4. Using decontaminated forceps and gloved fingers, fold filter paper into quarters and then roll (Figure 12). Keep filter stable and from unrolling by using gloved finger. Place filter in 2 mL vial filled with 1 mL ethanol (Figure 13).
5. Cap vial and label with sample site, number, and date, using an ethanol-proof marker.
6. Remove filter funnel from rubber stopper and discard.
7. Repeat for each sample, making sure to empty vacuum flask before it overfills.
8. Store sample vials at room temperature or colder, and away from light.

Note for shipping samples to a laboratory: Federal Express is currently the only major courier service that accepts ethanol in shipments.

Figure 11. Remove funnel cup.

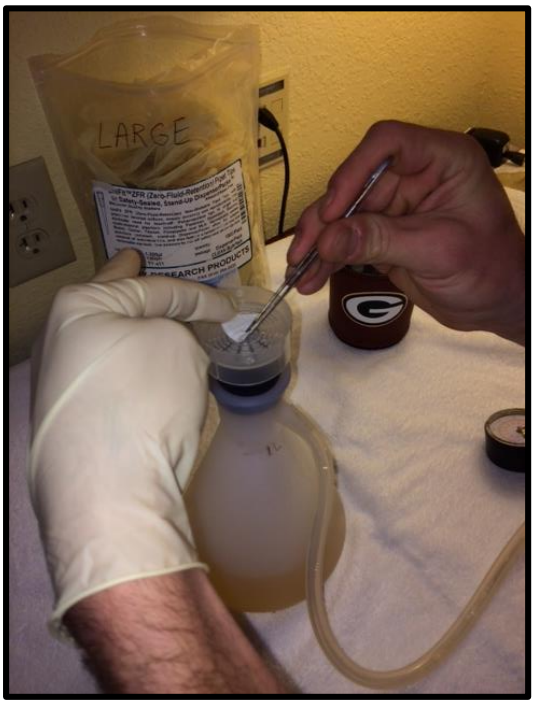

Figure 11. Fold filter.

Figure 12. Place filter in 2 mL tube of ethanol.